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Evaluation of the antifungal efficacy of *Mitracarpus scaber* extracts and *Ocimum gratissimum* oil against clinical isolates of fungi

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Abstract: Dermatophytes and *Candida albicans* are widespread with increasing prevalence and pose a grave threat to public health globally. *Ocimum gratissimum* and *Mitracarpus scaber* have been used in Southeastern Nigeria for several purposes, including antimicrobial effects, and have exhibited inhibition of growth to fungi. This study aimed to evaluate and ascertain the antifungal potential of these extracts against dermatophytes and *Candida albicans*. A total of 50 samples of dermatophytes and *Candida albicans* previously isolated from clinical samples at two different Hospitals, in the Southeast of Nigeria were used. The clinical samples were vaginal discharge, sputum, swab samples from the endocervix, urine, groin, mouth thrush, and palm. These organisms were all identified using standard mycological identification and characterization techniques. The sensitivity of selected fungi to the extracts (ethanolic extract of *M. scaber* and *O. gratissimum* oil) and ketoconazole was evaluated using a modified cup-agar diffusion plate method. The minimum inhibitory concentration (MIC) of the extracts was determined by the agar dilution method. Their minimum fungicidal concentration (MFC) and killing rates against the isolates were also determined. The organisms remain an important etiological agent in this species, implicated in several kinds of infections. The result showed that the activity of the *O. gratissimum* oil was comparable with that of the conventional drugs, indicating the potential in this extract. The MIC values for *Candida* isolates were consistently lower against *O. gratisimum* compared to ketoconazole. The MFC results indicated that *O. gratissimum* oil had a greater biocidal effect against most of the test organisms in contrast to ketoconazole. The killing rate study also indicated that the oil has very good activity against the isolates. *O. gratissimum* oil holds great potential for use in treating a wider spectrum of fungal pathogens.

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Introduction

Pathogenic fungi such as dermatophytes and *Candida albicans* cause superficial and severe systemic infections and are now widely recognized as essential agents of hospital-acquired infection [1]. These two organisms are widespread with increasing prevalence and stance a grave threat to public health globally [2-4]. Dermatophytes are superficial mycoses that cause tinea infections. They are keratinophilic moulds that infect human and animal skin, nails and hair and are typically confined to the superficial keratinized tissue [5]. They are of three genera namely: *Trichophyton, Microsporum* and *Epidermophyton* [6]. Infection can be present in children [7], but there is an indication of increase in infection with an increasing age [8, 9]. An increased incidence of dermatophyte infection in men compared to women is well known [10, 11]. As such, the tendency for men to develop dermatophytosis appears to be a widespread phenomenon. *Candida albicans* on the other hand is an opportunistic fungal pathogen of humans that colonizes the skin and mucosal surfaces of most healthy individuals. It causes superficial infections such as vaginal, oral and cutaneous candidiasis [12, 13] and more severe systemic infections like candidemia, endocarditis, endophthalmitis, pneumonia, septicemia, meningitis, osteomyelitis and fungal arthritis in individuals with compromised immune systems, leading to prolonged hospital stay and high mortality rate [14]. It is responsible for approximately one third to half of all nosocomial candida infections worldwide [15]. The focus of *Candida albicans* infection is the kidney [16]. For thousands of years now, plants have been seen as a valuable source of medicinal agents with proven potential for treating infectious diseases and with lesser side effects compared to the synthetic drug agents [17]. Medicinal plants have been used for several purposes including antimicrobial effects and have exhibited inhibition of growth to fungi. Some concerns have been expressed about the rising prevalence of pathogenic microorganisms that are resistant to the newer or modern antifungals [18]. There is a problem stood by the high cost and adulteration of these synthetic drugs [19] leading to a rising incidence of failures in the treatment of mycoses in the case of severely immunosuppressed patients, found more especially in the developing countries [20]. Thus, there is a need for an alternative therapy which is safer, cheaper, and more available to those in peculiar countries. Coincidentally, there is an increasing intensive study on extracts and biologically active compounds isolated from plant species used as natural herbal therapies [21]. Based on this, this study was aimed at evaluating and ascertaining the antifungal potentials of herbal extracts from *M. Scaber* and Oil from *O. gratissimum* against dermatophytes and *Candida albicans*. This study therefore, tested the susceptibility of isolated dermatophytes to local herbs used in the area for the treatment of these infections to ascertain their efficacy.

Materials and methods

Culture media: The culture media used were Sabouraud's Dextrose Agar (SDA) and Sabouraud Dextrose Broth (Middlesex-UK). These media were prepared according to the manufacturer's instructions.

Plant materials: Mitracarpus scaber and *Ocimum gratissimum* were collected from Nsukka, Enugu State and authenticated in the Department of Botany, University of Nigeria, Nsukka, Nigeria. Voucher specimens were deposited accordingly.

Extraction of plants: 500 g of Sun-dried powdered plant material of *Mitracarpus scaber* was extracted with 2000 ml of ethanol using the cold maceration method [22]. This filtrate was exposed to air until the solvent evaporated to dryness. The residue seen after drying (which is the extract from the plant) was collected, weighed, and kept in a container for further use.

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Volatile oil extraction: Fresh leaf samples were subjected to steam distillation in a modified Clevenger-type apparatus (Sunbim, India) for a minimum of three hours. The oil was obtained in a yield of 0.3% per 100 g, stored in a sealed glass vial and kept in a refrigerator at 4ºC until required.

Sample collection and processing: A total of 50 samples each of dermatophytes and *Candida albicans* previously isolated from clinical samples at University of Nigeria Teaching Hospital, and Federal Medical Centre Owerri, Southeast of Nigeria were used for the study. The clinical samples were scalp and skin for dermatophytes and vaginal discharge, sputum, swab samples from endocervix, urine, groin, mouth thrush, and palm. The isolates were inoculated onto Sabouraud dextrose agar plates containing 0.03% w/v chloramphenicol and incubated for 24 hrs and 7 days for *Candida albicans* and dermatophytes at 28ºC [23]. The colonial growths were stored in Sabouraud dextrose agar (SDA) slants containing 0.03% w/v chloramphenicol at 4ºC. Before use, an aliquot of the test isolates was cultured again onto fresh Sabouraud dextrose agar containing 0.03% w/v chloramphenicol and then incubated for 24 hrs and seven days for *Candida albicans* and dermatophytes at 28ºC for reactivation. Reactivated cultures were standardized by growing a 40 mm diameter of the mycelia growth into a 20.0 ml SDA plate and processed [for dermatophytes]. For *Candida albicans*, overnight (18 hrs) sub-cultures in sabouraud's dextrose broth were adjusted to 90.0% transmittance at 530 nm using distilled water [23].

Preliminary sensitivity testing of the isolates: The sensitivity of selected fungi to the herbal extracts (ethanolic extract of *M. scaber* and *O. gratissimum* oil) and ketoconazole were evaluated using a modified cup-agar diffusion plate method [24].

Evaluation of MIC of the two extracts and ketoconazole against fungi isolates using the agar dilution method: The MIC of the two herbal extracts in addition to ketoconazole were prepared separately in distilled water or DMSO. Each of these solutions was diluted two-fold serially with dilute DMSO up to eight dilutions. Thereafter, 1.0 ml from each dilution was seeded into 19.0 ml of molten sterile SDA and allowed to solidify. The plates were divided into eight segments and eight representative isolates (four isolates each of dermatophytes and *Candida albicans* selected based on their sources) were streaked in triplicates in each segment. The plates were then incubated for 48 hrs (*Candida albicans*) and five days (dermatophytes). Signs of growth were checked.

Evaluation of MFC of O. gratissimum oil and ketoconazole against fungi isolates: Based on the outstanding antifungal properties *O. gratissimum* oil extract exhibited, it was further selected for further testing in comparison with ketoconazole as the positive control. For the determination of MFC, the plates were further incubated for double the incubation period (four days for *Candida albicans* and 10 days for dermatophytes).

Killing rate evaluation of O. gratissimum oil and ketoconazole against fungal isolates. Stock solution (10 times the MFC) of the oil and ketoconazole that had a minimum fungicidal concentration was prepared in Sabouraud dextrose broth. One ml of this stock was added into 8.0 ml of Sabouraud dextrose broth and 1.0 ml of representative test organisms was added immediately. At various time intervals of 0.0 min, 20 min, 40 min, 60 min, 90 min, 120 min, 180 min, 6 hrs, and 24 hrs, 0.1 ml was withdrawn from the reaction mixture and diluted 100-fold in sterile normal saline. These various dilutions were plated in sterile SDA plates containing 0.03%w/v chloramphenicol and colonies were counted.

Results

Table 1 shows the different species of dermatophytes isolated from different sources causing skin infections. Both male and female ratio is shown with age ranging from 9 to 20 years old. Different species of dermatophytes were recovered from the hospital and the spectrum of causing skin infections is wide in this area. Different sources

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of isolates of *Candida albicans* recovered from palm, high vaginal swab, urine, Endocervical swab, Mouth thrush, sputum and groin. The inhibitory effects of the two extracts and ketoconazole against isolates of dermatophytes and *Candida albicans* are presented in **Table 2**. The MIC results of *M. scaber, O. gratissimum* oil extracts, and ketoconazole against selected fungal isolates are shown in **Table 3.** Based on the impressive MIC data exhibited by *O. gratissimum* oil, the MFC was further determined with ketoconazole as the positive control for comparative purposes. The minimum fungicidal concentration (MFC) results of ketoconazole and *O. gratissimum* oil against the isolates are presented in **Table 4**.

Table 1: Different species of dermatophytes isolated and their sources

Gender	Age	Source(s)	Isolates/ isolate number
Male	20	Skin/Scalp	*T. soudanense* (TS1) and *Cladosporium* spp (CS1)
Male	17	Skin/Scalp	*Trichophyton soudanense* (TS2)
Male	16	Skin/Scalp	*Cladosporium* spp (CS2)
Male	12	Skin/Scalp	*Penicillium* spp (PS1)
Male	11	Skin/Scalp	*Curvularia* spp (CSS1)
Male	11	Scalp	*Aspergillus niger* (AN1)
Male	12	Skin/Scalp	*Fusarium* spp (FS1)
Male	18	Skin/Scalp	*Trichophyton soudanense* (TS3)
Male	11	Skin scrapping	*Aspergillus niger* (AN2) and *A. flavus* (AF1)
Male	14	Skin scrapping	Mixed with the growth of *T. soundenense* (TS4) and *Cladosporium* spp (CS3)
Female	14	Skin	*Trichophyton soudanense* (TS5)
Female	11	Skin	*Aspergillus niger* (AN3)
Female	16	Skin	*Fusarium* spp (FS2)
Female	09	Skin	*Fusarium* spp (FS3)
Female	18	Skin	*Cladosporium* spp (CS4)
Male	19	Scalp	*Cladosporium* spp (CS5)
Male	19	Skin/Scalp	*Cladosporium* spp (CS6)
Male	10	Scalp	*Penicillium lilacinum* (PL1)
Female	19	Scalp	*Aspergillus flavus* (AF2) mixed with the growth of *Trichophyton mentagrophytes* (TM1)
Female	15	Skin	*Fusarium solani* (FSS1)

Table 2: Preliminary test on selected strains of *Candida albicans* and dermatophytes

Isolates	A (50 µg/ml)	B (50 µg/ml)	Keto (50 µg/ml)
1	65	14	20
2	44	15	20
3	64	15	14
10	70	15	-
11	54	-	30
12	60	14	25
13	60	13	20
14	55	10	-
15	53	-	33
18	64	1-	18
Trichophyton soudanense	54	15	35
Trichophyton mentagrophytes	55	15	35
Penicillium lilacinum	66	13	-
Fusarium spp	40	13	17
Cladosporium spp	50	14	20
Curvularia spp	40	15	18
Apergillus niger	61	15	18

A: O. gratissimum; B: Mitracarpus scaber; -, no growth; + - growth.

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Table 3: MIC of extracts and ketoconazole against selected isolates of dermatophytes and *Candida albicans*

Test organisms/Isolates	MIC (µg/ml)		
	M. scaber	Oil *O. gratissimum*	Keto
Trichophyton soundanense	40.00	0.625	1.25
Trichophyton mentagrophytes	20.00	0.625	5.00
Apergillus niger	20.00	1.25	2.50
Trichophyton soundanense	40.00	-	+
02	40.00	0.16	5.00
11	40.00	0.31	2.50
13	40.00	0.16	2.50
17	20.00	0.31	0.31

Oil - O. gratissimum oil; Keto – ketoconazole; + - growth; -, no growth.

Table 4: MFC of ketoconazole and oil against selected isolates of moulds and *Candida albicans*

Test organisms/Isolates	MIC (µg/ml)	
	Ketoconazole	Oil of *O. gratissimum*
Trichophyton soundanense	2.50	0.625
Trichophyton mentagrophytes	5.00	2.50
Apergillus niger	2.50	1.25
Trichophyton soundanense	2.50	0.156
02	+	0.156
11	2.50	2.50
13	5.00	0.625
17	1.25	0.625

The results of killing rate of *O. gratissimum* oil and ketoconazole against the isolates are presented in **Tables 5** to **8**. This is in line with the results of the MIC and MFC that were obtained earlier in **Tables 3** and **4**.

Table 5: Killing rate (total viable counts) of ketoconazole against dermatophytes

Test org./time (mins)	0	20	40	60	90	120	180	360	1440
T. soundanense	7.5	6	4	3	2	1	1	1	-
A. niger	8	6	5	3	2	2	2	1	-
T. mentagrophytes	9	8.5	3	2	2	1	1	-	-
T. soundanense	7	6	4	2	1	1	1	1	-

Table 6: Killing rate (total viable counts) of ketoconazole against *Candida albicans* over time

Isolates/time (mins)	0	20	40	60	90	120	180	360	1440
2	69	39	34	23	16	14	12	12	-
11	65	40	32	23	16	12	12	-	-
13	50	45	32	30	23	22	12	-	-
17	50	46	31	30	28	24	22	20	-

Table 7: Killing rate (total viable counts) of *O. gratissimum* oil against dermatophytes over time

Test Org./Time (mins)	0	20	40	60	90	120	180	360	1440
T. soundanense	44	34	15	4	-	-	-	-	-
A. niger	60	34	28	15	6	-	-	-	-
T. mentagrophytes	44	42	34	15	3	-	-	-	-
T. soundanense	80	62	48	44	53	73	47	30	-

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Table 8: Killing rate (total viable counts) of *O. gratissimum* oil against *Candida albicans* over time

Test org./time (mins)	0	20	40	60	90	120	180	360	1440
T. soundanense	44	34	15	04	-	-	-	-	-
A. niger	60	34	28	15	06	-	-	-	-
T. mentagrophytes	44	42	34	15	03	-	-	-	-
T. soundanense	80	62	48	44	53	73	47	30	-

Discussion

Fungal infections are emerging opportunistic infections that often occur in immunocompromised people and especially those living with HIV/AIDS. In these people, attacks commonly called mycosis are both superficial (skin) and invasive (systemic). These pathogenic fungi cause both superficial and serious systemic diseases and are now widely recognized as important agents of hospital-acquired infection [1]. Fungi implicated in these infections are molds and yeasts [25]. Fungal attacks are recurrent and therapeutic management is complicated by the emergence of multidrug resistant (MDR) fungi. This situation calls for research for new antifungal compounds with a broad spectrum of activity in addition to the existing molecules. One of the solutions is to explore traditional medicine to identify plants with interesting therapeutic properties, since medicinal plants have been used for several purposes and are known to inhibit the growth of several microorganisms including fungi. Among these plants *Mitracarpus scaber* and *Ocimum gratissimum* feature prominently.

This current research reveals the ease at which fungi isolates can be present in human body parts, as shown in **Tables 1** and **2**. The preliminary result shows that both antifungal extracts and the drug have activity against the fungal isolates tested. The activity of the *O. gratissimum* oil was comparable with that of the conventional drugs indicating the potential in this extract. This finding is consistent with the findings of other workers [26, 27]. Other findings revealed that the phenolic compounds isolated from *M. scaber* extracts are used for the treatment of skin infections caused by *Staphylococcus aureus* and *Candida albicans* [28, 29]. It has been previously shown that essential oils as well as compounds derived from *O. gratissimum* have a wide range of activities with the antimicrobial properties being the most studied [30]. Some work reported that 60% of essential oil derivatives examined to date were inhibitory to fungi while 30.0% inhibited bacteria [31].

Candida albicans still remains an important etiological agent in Southeastern Nigeria with this species implicated in several kinds of infections. As shown in **Table 3**, the MIC values for *Candida* isolates (isolates 1-3, 10-15, and 18) were consistently lower (0.16-5.0 ug/ml) against *O. gratisimum* compared to ketoconazole (1.25-5.0 ug/ml). This work is in contrast with the work done by Anejionu *et al*. [23], which revealed that *in vitro* antifungal activity of the ethanol extract of *Mitracarpus scaber* (50 ug/ml) showed that the clinical isolates were sensitive to the herbal extracts but were more sensitive to *O. gratissium* oil extract (MIC range of 0.8-1.25 ug/ml) than ketoconazole (MIC range of 0.31-5.00 ug/ml). The data from **Table 4** shows that *O. gratissimum* oil had a greater biocidal effect against most of the test organisms in contrast to ketoconazole. However, the MFC of ketoconazole against the test organisms indicated antagonism. This indicates that the effect of ketoconazole on the isolates is biostatic rather than biocidal; while that of *O. gratissimum* oil reveals more biocidal activity. This finding is in support with the studies of other researchers [32, 33]. The studies indicated that oil has very good activity against the isolates and kills them faster than ketoconazole especially against *Candida albicans*. This is an indication that the oil will be good for the treatment of *Candida albicans*. Notwithstanding the deviation of the total viable count in **Table 8**, where the total number of viable counts at 90 and 120 min are higher than others, there is still evidence that the oil has strong activity against dermatophytes, confirming the study done by Nwaneri *et al.* [33, 34]. The

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inhibitory activities of the extract have been attributed to the presence of hexadecanoic acid (37.21%), oleic acid (25.38%), and octadecanoic acid (16.19%) in the extracts of *O. gratissimum* [35]. The extracts of *O. gratissimum* have previously been discovered to have other antifungal properties. A component of *O. gratissimum* essential oil, called Tymol, was shown to be extremely effective against *T. rubrum, T. mentagrophytes, Candida neoformans, Candida albicans,* and *Malassezia pachydermatis* by Chand *et al*. [36]. Kinetic studies have been consistently used to quantify antimicrobial activity or interactions [37, 38]. In many ways, they appear to be superior when compared to other conventional methods of assessing antimicrobial interactions based on broth dilutions, agar diffusions or experimental animal infections.

Conclusion: This study demonstrates that the ethanolic extracts of *M. scaber* and *O. gratissimum* oil have antifungal activity against dermatophytes and *Candida albicans*. The activity of *O. gratissimum* oil was better than *M. scaber* extract and conventional antifungal drugs as shown by the *in vitro* susceptibility test data. The killing rate study indicated also that the oil has good activity against the isolates. This extract holds a great promise for use in treating a wider spectrum of fungal pathogens.

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